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SCIENCE DIRECT
Enzyme and Microbial Technology 38 (2006) 422–428
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Acidic and proteolytic digestion of α-amylases from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*: Stability and flexibility analysis

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Received 5 July 2004; received in revised form 6 June 2005; accepted 22 June 2005

Abstract

An investigation was carried out to compare the proteolytic resistance and acidic digestion of the mesophilic α-amylase from *Bacillus amyloliquefaciens* (BAA) and its thermophilic counterpart from *Bacillus licheniformis* (BLA). Correlation between sites of proteolytic cleavage and the three-dimensional structure of the α-amylases, with the application of theoretical modeling of BAA, allowed discussion of the flexibility and the stability of both enzymes. The thermophilic enzyme shows higher resistance to trypsin, papain and thermolysin but is sensitive to pronase and acidic digestion. Proteolytic digestion of the thermophilic enzyme leads to an increased activity of the enzyme at room temperature whereas results of SDS-PAGE indicate proteolytic cleavage. Furthermore, thermal stability and resistance to proteolysis for BLA and BAA in the presence of additives such as sorbitol, trehalose and glycerol were also investigated. In addition to thermal stabilization of the two enzymes, these additives also augmented the resistance of the enzymes to proteolysis.

Keywords: α-Amylase; Flexibility; Acid digestion; Additives; Stability; Protease

1. Introduction

During the last three decades, the starch-converting enzymes have been exploited by the starch processing industry as a replacement for acid hydrolysis in the production of starch hydrolysates and other applications. Currently, these enzymes comprise about 30% of the world's enzyme production [1]. α-Amylases are among the most important starch-converting enzymes. Two of the more commonly used α-amylases are those from *Bacillus licheniformis* (BLA) and *Bacillus amyloliquefaciens* (BAA). BLA is practically suited to industrial applications due to its thermal stability. Most other α-amylases, including those produced by *B. amyloliquefaciens*, are rapidly inactivated at temperatures in excess of 70 °C [2]. X-ray crystallography of BLA has been determined primarily in calcium-free form by Machius et al. [3] while the three-dimensional (3D) structure of BAA has not been elucidated to date. BLA has a central domain A formed by a $(\beta/\alpha)_8$-barrel, a large protrusion between β-strand 3 and α-helix 3 (domain B), and a C-terminal globular domain C consisting of a Greek key motif [3].

Limited proteolysis is widely used to probe flexible regions of the protein surface in the protein structure [4–6]. This is based on the hypothesis that limited proteolysis occurs exclusively at "hinge and fringes" [7] and conformational parameters such as accessibility and segmental mobility correlate quite well with limited proteolytic sites [4,5,9,10].

Abbreviations: BAA, α-amylase from *Bacillus amyloliquefaciens*; BLA, α-amylase from *Bacillus licheniformis*; r.m.s., root mean square

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0141-0229/$ – see front matter © 2005 Published by Elsevier Inc.
doi:10.1016/j.enzmictec.2005.06.021

According to the 3D structure of calcium-free form of BLA, five of such flexible areas have been identified [3]. These sites include regions V_{120}–I_{133}, K_{370}–R_{376}, R_{413}–S_{422}, V_{440}–T_{448} and E_{458}–W_{467}. When a protein structure is known, it is possible to explain proteolysis results. For this reason, the 3D structure of the mesophilic α-amylase is determined using modeling approach. Several recent studies comparing BLA and BAA suggest that stabilization could occur via induced extra flexibility in some parts of rigid structure of the thermophilic enzyme [11]. In this study, limited proteolysis and acid digestion of BLA and BAA have been compared and differences in local flexibility of the enzymes have been discussed. Also, the effects of additives including sorbitol, glycerol and trehalose on the resistance of BLA and BAA against proteolysis and acid digestion have been studied.

2. Materials and methods

α-Amylases (BAA and BLA), thermolysin, papain, pronase, trypsin, trehalose, 3,5-dinitrosalicylic acid (DNS), soluble potato starch, and Tris were purchased from Sigma (St. Louis, MO, USA). BLA was subjected to ion-exchange chromatography using DEAE-Sepharose and eluted with a linear gradient of 0–500 mM NaCl in Tris buffer, pH 7.5. All other chemicals were from Merck (Darmstadt, Germany) and were reagent grade.

2.1. Limited proteolysis of BLA and BAA

Digestion of BLA and BAA was performed by incubation of each protein (0.3 mg/ml) dissolved in 20 mM Tris buffer, pH 7.5 containing 10 mM $CaCl_2$ with thermolysin (0.03 mg/ml) and trypsin (0.03 mg/ml) in a separate reaction for 3 h. Limited proteolysis with papain (0.06 mg/ml) was carried out in 20 mM Tris buffer, pH 7.5 containing EDTA which is required for papain activity [12]. After 40 min, iodoacetamide has been used for stop the reaction of papain with proteins. Digestion with pronase (0.03 mg/ml) was performed in 50 mM Tris buffer, pH 7.5 containing 20 mM $CaCl_2$. At the end of the incubation time (1 h), aliquots were removed from the reaction mixture and immediately placed in sample buffer for SDS-PAGE. In the case of trypsin, and pronase aliquots were removed from reaction mixtures and phenylmethylsulfonylfluoride (PMSF), was added to a final concentration of 1 mM to inhibit further digestion and subsequently samples were immediately placed in sample solvent. SDS-PAGE was performed using 13% acrylamide gel according to Laemmli [13]. The gels were stained with coomassie brilliant blue R-250 and, when desired, scanned using a Hellena densitometer.

2.2. Determination of enzyme activity and protein concentration

Activity of α-amylase was determined at room temperature using potato starch (10 mg/ml) as a substrate in 20 mM Tris buffer, pH 7.5 containing 10 mM $CaCl_2$. The concentration of reducing sugars was obtained from the catalyzed reaction measured by the dinitrosalicylic acid method according to Bernfeld [14]. Protein concentration was determined by Lowry method [15].

2.3. Determination of thermal stability

The time course of irreversible thermoinactivation was measured by incubating BAA and BLA in 20 mM Tris buffer, pH 7.5 containing 10 mM $CaCl_2$ at 70 and 90 °C, respectively, in the presence and absence of glycerol, sorbitol and trehalose at 13% (v/v or w/v). At regular intervals, samples were removed, cooled on ice and the remaining activity was determined. Activity of the same enzyme kept on ice was considered as control (100%).

2.4. Digestion in acidic environment

Acidic digestion of BLA and BAA was performed by incubation of BLA and BAA (0.3 mg/ml) in 10 mM glycine buffer, pH 3. SDS-PAGE was carried out at different incubation times, after changing pH to neutral with immediate placing of aliquots in sample solvent. The effect of sorbitol, glycerol and trehalose on digestion of BLA and BAA was examined with incubation of enzymes in acidic pH in the presence and absence of each additive.

2.5. Size-exclusion chromatography

Size-exclusion chromatography of tryptolytic mixture was conducted at 37 °C on a G_{50} column (Pharmacia), eluted at a flow rate of 1.5 ml/min with 20 mM Tris buffer, pH 7.5. Effluent from the column was monitored at 280 nm. Following chromatography, SDS-PAGE analysis of fragments was performed using 13% acrylamide gel according to Laemmli [13].

2.6. Model building

BLASTP [16] search against PDB was used to obtain *B. amyloliquefaciens* α-amylase (BAA) homologues. *B. licheniformis* α-amylase (PDB code 1 bli) shows 80% identity (88% similarity) with BAA and therefore this is useful as the PDB template in web-based SWISS-MODEL protein modeling server and SWISS-PDB Viewer [17] to obtain 3D structure models of BAA. The fit between 3D structure of BLA (1 bli) and BAA was evaluated in SWISS-PDB Viewer by calculating the root mean square (r.m.s.) deviation after iterative fitting. Sequence similarity between BLA and BAA were calculated with BLAST 2 sequences [18]. Secondary structure of BLA and BAA model were obtained using STRIDE program [19]. Accessible surface area of both proteins was calculated by online GETAREA program [20].

Results presented in this paper are the mean from at least three repeated experiments in a typical run to confirm reproducibility.